(19) Eur päisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 648 126 B1

(12) EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention of the grant of the patent:
02.02.2000 Bulletin 2000/05

(21) Application number: **93912521.7**

(22) Date of filing: **16.06.1993**

(51) Int. Cl.[7]: **A61K 38/08**

(86) International application number:
PCT/CA93/00252

(87) International publication number:
WO 93/25224 (23.12.1993 Gazette 1993/30)

(54) PHARMACEUTICAL PREPARATIONS FOR INHIBITING TUMOURS ASSOCIATED WITH PROSTATE ADENOCARCINOMA, STOMACH CANCER AND BREAST CANCER

PHARMAZEUTISCHE ZUSAMMENSTEZUNGEN ZUR HEMMUNG VON TUMOREN IN VERBINDUNG MIT PROSTATEN ADENOKARZINOM MAGENKREBS UND BRUSTKREBS

PREPARATIONS PHARMACEUTIQUES D'INHIBITION DE TUMEURS ASSOCIEES A L'ADENOCARCINOME DE LA PROSTATE, AU CANCER DE L'ESTOMAC ET DU SEIN

(84) Designated Contracting States:
CH DE FR GB LI NL

(30) Priority: **16.06.1992 US 899535**

(43) Date of publication of application:
19.04.1995 Bulletin 1995/16

(73) Proprietor:
PROCYON BIOPHARMA INC.
London, Ontario N6L 1A8 (CA)

(72) Inventors:
- **SHETH, Anil, R.**
  Chowpatty, Bombay-400007 (IN)
- **GARDE, Seema**
  Worli, Bombay-400025 (IN)
- **PANCHAL, Chandra, J.**
  Lambeth, Ontario N0L 1S3 (CA)

(74) Representative:
Bannerman, David Gardner et al
Withers & Rogers,
Goldings House,
2 Hays Lane
London SE1 2HW (GB)

(56) References cited:
- **AMERICAN UROLOGICAL ASSOCIATION 87TH ANNUAL MEETING, WASHINGTON, D.C., USA, MAY 10-14, 1992. J.UROL. 147 (4 SUPPL.) 1992, page 335A 'Human Prostatic Inhibin suppresses growth of Dunning R3327-G Tumor in-vivo and inhibits Tumor Cell Colony in-vitro'**
- **THE PROSTATE vol. 22, no. 3, 1993, pages 225 - 233 'Effect of Prostatic Inhibin Peptide (PIP) on Prostate Cancer Cell Growth in Vitro and in Vivo'**
- **NATURE vol. 360, no. 6402, pages 313 - 19 'Alpha-inhibin is a tumor-suppressor gene with gonadal specificity in mice'**
- **DNA,6(1) 1987,23-29**

EP 0 648 126 B1

Description

FIELD OF THE INVENTION

[0001] The present invention relates to pharmaceutical preparations for use as tumour suppressive agents for tumours arising from prostatic adenocarcinoma, stomach cancer, breast cancer and benign prostatic hyperplasia.

BACKGROUND OF THE INVENTION

[0002] The prostate gland, which is found exclusively in male mammals, produces several components of semen and blood and several regulatory peptides. The prostate gland comprises stroma and epithelium cells, the latter group consisting of columnar secretory cells and basal nonsecretory cells. A proliferation of these basal cells as well as stroma cells gives rise to benign prostatic hyperplasia (BPH) which is one common prostate disease. Another common prostate disease is prostatic adenocarcinoma (CaP) which is the most common of the fatal pathophysiological prostate cancers and involves a malignant transformation of epithelial cells in the peripheral region of the prostate gland. Prostatic adenocarcinoma and benign prostatic hyperplasia are two common prostate diseases which have a high rate of incidence in the aging human male population. Approximately one out of every four males above the age of 55 suffers from a prostate disease of some form or another. Prostate cancer is the second most common cause of cancer related death in elderly men with there being approximately 96,000 cases diagnosed and about 26,000 deaths reported annually in the United States.

[0003] Studies of the various substances synthesized and secreted by normal, benign and cancerous prostates carried out in order to gain an understanding of the pathogenesis of the various prostate diseases reveal that certain of these substances may be used as immunohistochemical tumour markers in the diagnosis of prostate disease. The three predominant proteins or peptides secreted by a normal prostate gland are Prostatic Acid Phosphatase (PAP), Prostate Specific Antigen (PSA) and Prostatic Inhibin Peptide (PIP) also known as Human Seminal Plasma Inhibin (HSPI) and hereinafter referred to as HSPI.

[0004] Metabolic and immunohistochemical studies have shown that the prostate is a major source of HSPI. HSPI is involved in the feedback control of, and acts to suppress secretion of, circulating follicle stimulating hormone (FSH) both in-vitro and in-vivo in adult male rats. HSPI acts both at the pituitary as well as at the prostate site since both are provided with receptor sites for HSPI.

[0005] Both PSA and PAP have been studied as tumour markers in the detection of prostate disease but since both exhibit elevated levels in prostates having benign prostatic hyperplasia (BPH) neither marker is specific and therefore they are of limited utility.

[0006] Recently, it has been shown that HSPI concentrations in serum of patients with BPH or CaP are significantly higher than normal. The highest serum concentration of HSPI observed in normal men is approximately 40 ng/ml., while in men with either BPH or CaP serum concentrations of HSPI have been observed in the range from 300-400 ng/ml. Because there exists some overlap in the concentrations of HSPI in subjects having normal prostates and patients exhibiting either BPH or CaP, serum levels in and of themselves are of little value.

[0007] A major therapy in the treatment of prostate cancer is androgen-ablation. While most patients respond initially to this treatment, its effectiveness decreases over time possibly because of the presence of a heterogenous population of androgen-dependant and androgen-independent cells to begin with. In such a scenario, the androgen sensitive cells respond to the androgen treatment while any androgen insensitive cells present would continue to proliferate unabated.

[0008] Other forms or cancer which are currently exacting a heavy toll are breast cancer in women and cancer on the gastrointestinal tract. Currently, the use of various cancer drugs such as mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin and daunomycin form part of the therapy for treating such cancers. One drawback to such a therapeutic treatment is the presence of adverse side effects due to the drugs in the concentration ranges required for effective treatment.

[0009] Accordingly, it would be advantageous to find a more effective means of arresting the growth of prostate, breast and gastrointestinal cancer cells and tumours which can be used effectively against both androgen sensitive and androgen insensitive cells.

[0010] The provision of compositions of prostatic inhibin in isolation for treating prostatic carcinoma has been reported, J.Urol. Vol. 147, (4.Suppl.), 1992, page 335A.

[0011] The cloning of seminal plasma protein is reported in DNA, Vol. 6, No. 1, 1987, pages 23-29.

SUMMARY OF THE INVENTION

[0012] A first aspect of the invention provides a composition for use in the medical treatment of benign prostatic hyperplasia, adenocarcinoma of the prostate, breast or gastrointestinal tract, comprising:

in combination an anticancer drug and a peptide selected from human seminal plasma inhibin (Sequence ID No. 1) and a decapeptide (Sequence ID No. 2).

[0013] A second aspect of the invention provides a composition for use in the treatment of diseases characterised by elevated levels of FSH, comprising:

in combination an anticancer drug and a peptide selected from human seminal plasma inhibin (Sequence ID No. 1) and a decapeptide (Sequence ID No. 2).

[0014] Preferably, said anticancer drug is selected from mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin and daunomycin.

LIST OF TABLES

[0015]

Table I summarizes data showing the effect of HSPI administration on the serum levels of FSH and LH (ng/ml^{-1}) in intact adult male rats;
Table II summarizes data showing the effect of HSPI on cell proliferation;
Table III summarizes data showing the effect of HSPI on the weight (grams) of testes and prostate;
Table IV summarizes in-vivo data relating to HSPI dosage levels and subsequent tumour viability; and
Table V summarizes data on various hormone levels measured in rats 14 days after treatment with two different levels of HSPI.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] The invention will now be described, reference being had to the drawings, in which:

Figure 1 shows the complete sequence of HSPI (Sequence ID No.1);
Figure 2 illustrates an HPLC profile of HSPI on a gel permeation column (LKB-TSK G 3000 SW 7.5x600 mm), the material being eluted as a major peak;
Figure 3 illustrates a reverse phase HPLC of purified HSPI on a column of Lichrosorb RP-18 (5μm;0.4x25 cm, eluant, A 0.1% (w/v) aqueous TFA; B, 50% CH_3CN in 0.1% aqueous TFA; Flow rate, 1 ml/min, inset: SDS gel electrophoresis pattern of purified HSPI (method of Laemmli, 1970);
Figure 4 displays the % survival of Dunning tumour R-3327-G cell lines on concentration of HSPI (Sequence ID No. 1);
Figure 5 displays the % survival of Dunning tumour R-3327-G cell lines on concentration of the decapeptide analogue (Sequence ID No.2);
Figure 6 shows the effect of various concentrations of HSPI (Sequence ID No. 1) on the growth of R-3327-G cells;
Figure 7 displays the patterns of DNA synthesis in R3327-G cells treated with and without HSPI, as depicted by ^3H-thymidine incorporation;
Figure 8 displays the effect of HSPI (Sequence ID No. 1) on tumour volume in Dunning rats;
Figure 9 shows a comparison of tumour volume as a function of duration of treatment with saline, leuprolide and HSPI (Sequence ID No. 1);
Figure 10 summarizes studies of the effect of FSH on prostate cancer cell growth in-vitro and its inhibition by HSPI;
Figure 11 illustrates the R-10 peptide (Sequence ID No. 2) in the box which are the last 10 amino acids of HSPI (Sequence ID No. 1) but with lysine in position 85 replaced by tyrosine and with isoleucine in position 93 replaced by glycine;
Figure 13 summarizes the data of Table IV in bar graph form;
Figure 14 illustrates the cytotoxic effect of HSPI (Sequence ID No.1) with and without the anticancer drug idarubicin on the human gastric cancer cell line;
Figure 15 illustrates the cytotoxic effect of HSPI (Sequence ID No.1) with and without the anticancer drug daunomycin on the human gastric cancer cell line;
Figure 16 illustrates the cytotoxic effect of HSPI (Sequence ID No.1) with and without the anticancer drug adriamycin on the human gastric cancer cell line;
Figure 17 illustrates the cytotoxic effect of HSPI (Sequence ID No.1) with and without the anticancer drug cisplatin on the human gastric cancer cell line;
Figure 18 illustrates the cytotoxic effect of HSPI (Sequence ID No.1) with and without the anticancer drug methotrexate on the human gastric cancer cell line;
Figure 19 illustrates the cytotoxic effect of HSPI (Sequence ID No.1) with and without the anticancer drug 5-fluoro-uracil (5-FU) on the human gastric cancer cell line; and

Figure 20 illustrates the cytotoxic effect of HSPI (Sequence ID No.1) with and without the anticancer drug mitomycin on the human gastric cancer cell line.

DESCRIPTION OF THE INVENTION

[0017] The inventors have considered that high levels of HSPI (Sequence ID No.1) under pathophysiological conditions associated with prostate cancer may serve as a form of defence mechanism, albeit apparently not always effective, which may be initiated by the prostate. Various in-vivo and in-vitro experimental studies have been carried out and are summarized herebelow to determine the efficacy of concentrations of HSPI (Sequence ID No.1) higher than concentrations secreted by the diseased prostate as tumour suppressive agents for arresting or inhibiting the growth of prostatic adenocarcinoma. Studies have also been carried out to determine the efficacy of a synthetic analogue of HSPI (Sequence ID No.1), specifically a peptide having 10 amino acids (Sequence ID No.2), as a tumour suppressive agent. This synthetic analogue has been shown to closely mimic the action of HSPI (Sequence ID No.1) in suppressing circulating FSH levels preferentially without altering the levels of luteinizing hormone (LH).

[0018] The bar graph of Figure 10 summarizes studies of the effect of FSH on prostate cancer cell growth in-vitro and its inhibition by HSPI (Sequence ID No.1). The tumour cells were exposed for 48 hours to HSPI with 0.5% serum in tissue cultures.

PREPARATION OF HSPI (Sequence ID No.1)

[0019] Referring to Figure 1, HSPI (Sequence ID No.1) is a simple nonglycosylated protein comprising at least 94 amino acid residues. HSPI (Sequence ID No.1) is produced by the prostate.

[0020] HSPI antigen was purified according to the basic procedure of Thakur et al. (1981) ISOLATION AND PURIFICATION OF INHIBIN FROM HUMAN SEMINAL PLASMA, Indian Journal of Experimental Biology, 19, 307-313 but with modifications (Thakur et al. and Sheth et al. (1984) CHARACTERIZATION OF A POLYPETIDE FROM HUMAN SEMINAL PLASMA WITH INHIBIN (INHIBITION OF FSH SECRETION) -LIKE ACTIVITY, FEBS Letters, 165, 11-15.). Sperm-free human seminal plasma was precipitated with alcohol (1:4 vol/vol) and then extracted with 0.05 M acetate buffer, pH 4.0. The soluble proteins were separated using chromatography on a Sephadex G-100 column (3.5x100 cm) using 0.01 M acetate buffer for equilibrium and elution. The fraction with FSH-sappressing activity was subjected to ion-exchange chromatography on DEAE-cellulose (3x30 cm). The column was washed initially with 0.05 Tris buffer, pH 8.0. The bound material was eluted using an NaCl gradient (0-0.5 M) in the same buffer. The active material collected was subsequently purified by high pressure liquid chromatography (HPLC) using a gel permeation column (LKB-TSK G-3000 S.W., 7.5 x 600 mm) and 0.01 M acetate buffer, pH 4 for equilibration and elution, see Figure 2.

[0021] The HPLC purified material exhibited a single band on SDS-Gel electrophoresis at pH 8.3 (see inset of Figure 3). On reverse phase HPLC, the purified material eluted as a single peak, see Figure 3.

[0022] The fractions obtained at each stage of purification were assayed for bioactivity using intact adult male rats. The assay is based on suppression of circulating FSH levels. Administration of HPLC-purified HSPI (Sequence ID No.1) to adult male rats for 3 consecutive days caused specific suppression of circulating FSH levels, see Table 1. No significant change in LH levels was observed.

SYNTHESIS OF DECAPEPTIDE HSPI ANALOGUE

[0023] The decapeptide (Sequence ID No.2) analogue of HSPI (Sequence ID No.1) forming part of the subject invention disclosed herein is a synthetic analogue of the 85-94 amino acid residues at the carboxyl terminal of the HSPI (Sequence ID No. 1) sequence. The decapeptide (Sequence ID No. 2) differs from HSPI (Sequence ID No.1) in that the lysine residue at position 85 in HSPI is replaced by a tyrosine residue in position 1 in the decapeptide and the cysteine residue at position 3 in the decapeptide is protected by an acetamidomethyl group. The Ile residue at position 93 of HSPI is replaced by Gly in the corresponding position 9 of the decapeptide. This synthetic decapeptide (Sequence ID No. 2) and other peptide sequences were prepared using an Automated Peptide Synthesizer.

IN-VITRO AND IN-VIVO STUDIES

[0024] Studies were carried out using the rat Dunning R-3327-G tumour which is a pre-eminent animal model for the study of CaP. The Dunning tumour is a fast growing, poorly differentiated, transplantable tumour which can be maintained both in-vivo in the Copenhagen x Fisher 344 rat and in-vitro as a cell line.

EXPERIMENT 1

EFFECT OF HSPI ON IN-VITRO CELLS

[0025] Dunning tumour R-3327-G lines derived from cells dissociated in their 20th and 28th in-vivo passages in Copenhagen x 344 male carrier rats were used for the in-vitro studies. Tumours were excised and dissociated into single cells and cultured in T-25 culture flasks (Corning N.Y). Dissociated tumour cells were dislodged from the culture flask by trypsinization (0.25% trypsin and 0.02% EDTA at 37°C for 3 minutes) and passaged in alpha-MEM (GIBCO Labs, Grand Island, N.Y.) supplemented with 2 mM L-glutamic acid, 20% fetal bovine serum (FBS, Hyclone Labs., Logan, V.T.) and antibiotics (complete medium = CM). Cultures were passaged every five days.

[0026] For colony assay, R-3327-G cells between 2 and 10 in-vitro passages were trypsinized, dispersed into single cell suspension and cultured in 35 mm tissue culture dishes at 0.5 - 1.0 x 10^1 viable cells in 2ml CM. HSPI (Sequence ID No.1) was diluted at various concentrations in CM, filtered, sterilized and then added at appropriate concentration to culture dishes. These culture dishes were incubated in a humidified incubator at 37°C with 5% CO_2 for seven days. Following this the culture dishes were emptied, washed twice in cold phosphate buffer saline (PBS) solution and then fixed in absolute methanol for 5 minutes. The culture dishes were then stained with acidified Harris Hematoxylin and the colonies were counted manually.

[0027] Between about 20% to 30% of plated R-3327-G cells formed characteristic diffuse colonies within 7 days. Typically, colonies consisted of 102.3 ± 13.7 cells. A dose dependent inhibition of both colony number and colony size were observed with addition of various concentrations of HSPI (Sequence ID No.1). Above concentrations of 100 ng/ml the colony inhibition was significant, leading to a 50% reduction at a HSPI (Sequence ID No. 1) concentration of 1 µg/ml. Increasing concentrations of HSPI (Sequence ID No. 1) resulted in small cell-clusters (50 cell-Figure 4). Replenishing the culture media along with the HSPI (Sequence ID No.1)on the 4th day of the culture resulted in more effective and consistent inhibition of colony growth than that of one time HSPI (Sequence ID No.1) addition.

EXPERIMENT 2

EFFECT OF DECAPEPTIDE ON IN-VITRO CELLS

[0028] The synthetic decapeptide (Sequence ID No.2) shown in the box in Figure 11 has been shown to mimic the biological action of HSPI (Sequence ID No.1) and therefore its effect on the R-3327-G cells was studied. Referring to Figure 5, the decapeptide (Sequence ID No. 2)has a similar inhibitory action as HSPI (Sequence ID No.1) on in-vitro R-3327-G cell culture. Specifically, a 50% colony count inhibition was observed with 50 ng/ml of the decapeptide (Sequence ID No.2) leading to a maximum of 70% inhibition at 1 µg/ml. However, referring again to Figure 4, an equimolar concentration of HSPI (Sequence ID No. 1) was found to have a greater inhibiting effect compared to the decapeptide.

EXPERIMENT 3

EFFECT OF HSPI ON ANDROGEN DEPENDENT AND INDEPENDENT R-3327-G IN-VITRO CELL COLONIES

[0029] The R-3327-G tumours comprise both androgen sensitive and androgen insensitive cells. The effect of HSPI (Sequence ID No.1) on these two cell populations was studied in-vitro. Cells were dissociated from a R-3327-G tumour in its 20th in-vivo passage and were cultured in the presence or absence of steroids. For comparison, cells from the 28th in-vivo passage known to be largely androgen insensitive were cultured in the same way.

[0030] The results of the effect of various concentrations of HSPI (Sequence ID No.1) on the in-vivo cells is summarized in Figure 6. The effect of HSPI (Sequence ID No.1) was similar under all test conditions for both androgen sensitive and androgen insensitive cells. Although the actual number of colonies which appeared under each assay condition were different with these cells, the extent of HSPI (Sequence ID No.1) induced colony inhibition was comparable in all.

EXPERIMENT 4

INHIBITION OF CELL-GROWTH BY HSPI

[0031] Colony inhibition might occur as a result of immediate cell death or due to delay in the cell cycle. In order to distinguish between these two routes of inhibition, the following experiment was conducted.

[0032] Aliquots of 0.5 x 10^1 cells were cultured in 24 well plates and incubated with various concentrations of HSPI (Sequ nce ID No.1). Cell counts were taken on days 3 and 7. In control wells the number of cells increased 4-fold after

3 days and 28-fold after 7 days. At a dose of HSPI (Sequence ID No.1) of 1 µg/ml, no increase in cell number was observed on day 3 while only a 5-fold increase was observed on day 7, see Table II.

[0033] The results of this study were further corroborated by measuring DNA synthesis using ^{3}H-thymidine. Specifically, R-3327-G cells were cultured in 24-well tissue culture plates (Costar, MA) in the presence or absence of HSPI (Sequence ID No.1) for six days. ^{3}H-thymidine (68 Ci/mmole, ICM Ca) diluted in CM containing 10 µM thymidine (Sigma MO) was added to duplicate culture wells (0.5 µCi/ml). Plates were further incubated for 18 hrs. The amount of ^{3}H-thymidine incorporated was estimated by precipitation with trichloro acetic acid, as described previously. Figure 7 shows patterns of DNA synthesis in R-3327-G cells treated with and without HSPI, as depicted by ^{3}H-thymidine incorporation. Cultures that received HSPI (Sequence ID No. 1) in the amount of 1 µg/ml had incorporated by day 7 only about 20% of radioactivity as compared to that of the control. The inhibitory effect of the HSPI (Sequence ID No. 1) was more pronounced on day 7 than on day 3.

EXPERIMENT 5

IN-VIVO EXPERIMENT

[0034] Copenhagen x Fisher 344 F hybrid male rats were ear-tagged and implanted with R3327-G cells ($1x10^1$ cells/animal in the 28th in-vivo passage) as described earlier. The animals weighed approximately 500 grams at the time of tumour implantation. A treatment regimen was initiated when tumour volume measured 0.2 to 0.5 cc.

[0035] Tumour bearing animals were divided into two groups of eight. One group comprising the control group, received saline injection while the other group received HSPI (Sequence ID No. 1) dissolved in saline in the amount of 5 µg/kg subcutaneously every day.

[0036] The tumour volume was approximated by 3-dimensional measurement using the formula 0.5236 x length x width x depth. The rats were sacrificed 24 days after tumour implantation as control tumours at that point in time started becoming necrotic. Accessory sex organs and tumours were excised from the rats and weighed.

[0037] Significantly reduced tumour growth was observed in animals treated with HSPI (Sequence ID No. 1) as compared to that of the saline group. Referring to Figure 8, the difference between the tumour volume in the control group and the HSPI (Sequence ID No. 1) treated group became increasingly pronounced with longer treatment. As tumours in the control group started to become necrotic on day 24, tumour and accessory sex organs were excised and weighed on this day. Mean tumour weight of the HSPI-treated group was 2.66 ± 0.48 g as compared to 6.44 ± 1.19 g for the saline treated control group. A 58% reduction in tumour weight was observed at the end of the experiment i.e. on the 24 day following tumour implantation or on the 10th day following administration of HSPI (Sequence ID No. 1) as compared to the saline treated control group. No significant change was observed in testes weight and prostate weight in HSPI (Sequence ID No. 1) treated group, see Table III.

EXPERIMENT 6

IN-VIVO EXPERIMENT

[0038] The tumour bearing animals were divided into three group of 8 animals. The first group was the control group and received saline treatment. The second group received HSPI (Sequence ID No. 1) in the amount of 5µg/kg and the third group received 1000 µg Leuprolide™/kg. This treatment regimen continued until the tumour volume for each animal reached approximately 10 cc. Tumour volumes were measured twice a week as described earlier.

[0039] The tumour volume data for each individual tumour was log transformed. Statistical analysis between treated and control group was performed by student "t" test.

[0040] As these results clearly demonstrated a growth inhibition following administration of HSPI (Sequence ID No. 1), the study was further extended to estimate tumour growth delay in HSPI-treated animals. Most of the tumours become necrotic by the time they reach 10 cc volume, following which the measurements may not be accurate thus, keeping this in mind, 10 cc was taken as an end point in this study. Among 8 animals in the treated group, tumour volume in 6 reached 10 cc by day 42 and in 2 by day 38. In the saline control group, tumour volume reached this size by day 30, see Figure 9. In other words, a delay of 10 days in tumour growth was observed in the HSPI-treated animals. In all experiments the difference in tumour growth rate curves of treated and control groups of animals was similar.

[0041] The cells used for the foregoing experiment were from the 28th in-vivo passage, which is a poorly differentiated androgen-insensitive tumour. In order to confirm this earlier observation, one group of animals were treated with Leuprolide which is an anti-androgen. There was no significant difference in the tumour growth rate of Leuprolide-treated animals as compared to the saline control group.

EXPERIMENT 7

IN-VIVO EXPERIMENTS USING MAT-LYLU CELL LINES

[0042] The androgen independent Dunning rat adenocarcinoma cell lines, Mat-Lylu were obtained from Dr. J. T. Isaacs, Johns Hopkins Medical School, Baltimore, Maryland and cultured in the laboratory by using RPMI 1640 medium containing 10% fetal calf serum and 1% antibiotics. When the cells reached confluency, they were trypsinized, dispersed into single cell suspension and the cell count was taken using hemocytometer.

[0043] Tumours were induced in adult Copenhagen male rats weighing about 200 gms by subcutaneous injection of $2X10^6$ cells on two sides of the flank area. Animals were segregated into different groups and the HSPI (Sequence ID No. 1) injections were initiated on day 4 following the induction of tumour growth. Table IV shows the various concentrations of HSPI (Sequence ID No. 1) injected into the animals.

[0044] Animals were injected every day and sacrificed on day 14 following the administration of tumour cells. The body weight and the tumour weights were recorded for both control and treated groups. Blood was collected through a cardiac puncture and serum FSH, LH, prolactin, testosterone and HSPI (Sequence ID No. 1) were measured by radio immunoassay. These serum levels of the above mentioned hormones are summarized in Table V for the control group and animals treated with dosages of 5 ng and 50 ng of HSPI (Sequence ID No. 1). These results show that FSH levels decrease with dosage which suggests the mechanism of action of HSPI (Sequence ID No. 1) relates to levels of FSH. In addition, testosterone levels are not adversely affected which indicates no loss of libido, in contrast to libido loss observed with current drugs used in the treatment of prostate and other forms of cancer.

[0045] A piece of tumour tissue from each animal was fixed in 10% buffered formalin to study the morphology of the cells. Table V shows the % viability of the tumours in treated groups when compared to the controls (100%). The results of table IV are summarized in the bar graph of Figure 13.

[0046] The foregoing studies show that HSPI (Sequence ID No. 1), when administered in a predetermined concentration range, results in a significant inhibition, in-vivo of cancerous tumours associated with the prostate. Specifically, the Dunning rat studies with HSPI (Sequence ID No. 1) show that an effective drug dosage "window" of between about 5 ng to 500 ng per 200 grams body weight exists. These results have been corroborated by several studies.

[0047] Those skilled in the art will be aware of the methods of preparing pharmaceutically appropriate dosage forms for HSPI (Sequence ID No.1) as applied to humans. Those skilled in the art will also appreciate that such dosages may be encapsulated and delivered using slow release technology comprising for example a liposome delivery system, polysaccharides exhibiting a slow release mechanism, salistic or other polymer implants or microspheres.

EXPERIMENT 9

STUDY OF THE EFFECT OF HSPI ON FRESH GASTRIC TUMOUR CELLS IN-VITRO BY METHYL TETRAZOLIUM SALT (MTT) ASSAY

[0048] Gastric tumour specimens were collected from patients with stomach cancer undergoing gastrectomy at Tata Memorial Hospital. Tumour specimens were collected in sterile DMEM and immediately transferred to the laboratory under cold conditions. The gastric tumour specimens were finely minced with a sterile pair of scissors. The finely minced gastric tissue was incubated with 1% collagenase 1 and IV in Dulbeco's minimum essential medium (DMEM) with 10% fetal calf serum (FCS) at 37°C with 5% CO_2 in an incubator for 1 hr. The whole mixture was then passed through a Millipore filter assembly and wire mesh (30 μm size) to get a single cell suspension of gastric tumour cells. The cells obtained were further subjected to primary culture in sterile culture bottles in 50 ml DMEM with 10% FCS and incubated for 12-18 hr. at 37°C with 5% CO_2 in an incubator, with 10 μl of 0.1, 0.5, 1.0, and 5.0 μg/ml concentration of HSPI (Sequence ID No.1) in a sterile 96 well microtitre plate. Blank and control in 6 microwells each were run along with tests. The plate was further incubated for 48 hrs. at 37°C in 5% CO_2. After 48 hrs., 10 μl of 5 mg/ml MTT was added in each well. After 6 hrs. of incubation at 37°C, 100 μl of 1 N HCl: Isopropanol (1:25) was added to each well and mixed vigorously to dissolve the farmazan crystals. Absorbance values at 540 nm were determined on an ELISA reader. Blank values were subtracted from the control and test values.

[0049] The percentage cell survival for each concentration of HSPI (Sequence ID No.1) along with concentrations of known 1) anticancer drugs used in the treatment of gastric cancer including cisplatin, 5-fluorouracil, methotrexate, mitomycin, and 2) other anticancer drugs used in chemotherapy including idarubicin, adriamycin, doxorubicin, and daunomycin and combinations of HSPI (Sequence ID No.1) and these anticancer drugs were calculated and compared to control. The results of these studies are summarized in Figures 14 to 20. As these results show, HSPI (Sequence ID No.1) by itself acts as a cytotoxin for stomach cancer cells. However, HSPI (Sequence ID No. 1) used in combination with the various anticancer drugs gives rise to a significantly enhanced cytoxic effect on cancerous cells as illustrated in Figures 14-20. The symbiotic effect obtained with the various combinations is evid nced by comparison to the pure

HSPI (Sequence ID No. 1) and anticancer drugs. It is anticipated that there will be an increased therapeutic effect. Specifically, as a significantly increased growth inhibitory effect is obtained with the above disclosed combinations utilizing lower concentrations of the anticancer drugs compared to the treatment regimes in which the drugs are used alone, there is the potential to provide therapy wherein adverse side effects associated with the anticancer drugs are considerably reduced than normally observed with the anticancer drugs used alone in larger dosages.

[0050] The applicability of HSPI, the peptide sequence (Sequence ID No. 2) demonstrating an efficacy for suppressing FSH levels and combinations of HSPI (Sequence ID No. 1) and this sequence with known anticancer drugs for the treatment of various cancers found in mammals such as prostate cancer breast and gastrointestinal cancer will be readily apparent to those skilled in the art. Further, the use of HSPI (Sequence ID No. 1), the shorter peptide (Sequence ID No. 2) and combinations thereof for treatment of benign prostate hyperplasia will also be apparent to those skilled in the art. The studies disclosed herein are interpreted to mean that HSPI (Sequence ID No. 1), the shorter peptide (Sequence ID No. 2) and combinations thereof with various anticancer drugs will exhibit an efficacy in the treatment of diseases characterized by elevated levels of FSH in the body.

[0051] Various amounts of HSPI (Sequence ID No. 1) in the range of 10 - 50 μg have been administered to adult male rats for a period of 4 to 12 weeks with no adverse toxic effect on body weight, or in parameters measured by clinical chemistry.

[0052] Those skilled in the art will be aware of pharmaceutically appropriate dosage forms for the mixtures of HSPI (Sequence ID No.1) and the anticancer drugs as well as the manner in which a suitable dosage quantity and regimen may be derived in respect of a particular patient suffering from cancer of the gastrointestinal tract. In addition, those skilled in the art will also appreciate that such dosages may be encapsulated in time release delivery systems comprising for example a liposome delivery system, polysaccharides exhibiting a slow release mechanism, salistic or other polymer implants or microspheres.

[0053] While HSPI (Sequence ID No, 1) and the peptide analogue (Sequence ID No. 2) associated therewith, and combinations of HSPI and this peptide analogue with anticancer drugs has been disclosed herein as exhibiting an efficacy for the treatment of prostate cancer and cancer or the gastrointestinal tract, it will be appreciated by those skilled in the art that numerous variations exist with respect to therapeutically treating various cancers characterized by elevated FSH levels with HSPI (Sequence ID No. 1), the analogue (Sequence ID No. 2) and combinations of these compounds with various anticancer drugs.

TABLE I

Effect of HSPI administration on the serum levels of FSH and LH (μg ml^{-1}) in intact adult male rats.

	FSH				LH			
	Saline	**100 ng**	**1 μg**	**10 μg**	**Saline**	**100 ng**	**1 μg**	**10 μg**
Mean± SEM	349	267.4*	223.7*	132*	402.2	398	386	351
(n = 5)	±20.8	±10.9	±10.2	±12.1	±28.6	±15.6	±30.3	±21.2
%Suppression	--	19.4	32.6	60.2	--	2.4	5.1	11.5

*P<0.001, in comparison with saline control.

HSPI (Sequence ID No. 1) was administered (s.c.) daily for 3 days, and blood collected 2 h after the last injection.

Table II

Effect of HSPI on cell Proliferation

	TREATMENT		CELL COUNT	
	CELLS/WELL	**3 DAYS INHIBITION(%)**	**CELLS/WELL**	**7 DAYS INHIBITION(%)**
CONTROL	2150	0	1.44×10^4	0
10/μg	330	84	0.28×10^4	80
5/μg	1165	45	0.708×10^4	50

Table II (continued)

Effect of HSPI n cell Proliferati n				
	TREATMENT		CELL COUNT	
	CELLS/WELL	3 DAYS INHIBITION(%)	CELLS/WELL	7 DAYS INHIBITION(%)
1/µg	2000	7	0.958×10^4	30

[0054] R-3327 (G) cells were seeded at a cell density 500 cells/well in 16 mm multiwell plates in MEM supplemented with 15% EBS. Different concentrations of HSPI was added as indicated. One plate was counted on day 3 while other plate was supplemented with indicated amount of HSPI and cell counts were carried out 7 days after initial addition of HSPI. Percentage inhibition was calculated taking control as 100% values are means of triplicate.

Table III

Effect of HSPI on Weight of Testis and Prostate Weight (grams)		
	Testis	Prostate
Saline Control	3.26 +/- 0.19	1.26 +/- 0.24
HSPI Treated	3.56 +/- 0.31	1.11 +/- 0.21

TABLE IV

GROUPS HSPI DOSAGE	% VIABILITY WHEN COMPARED TO CONTROLS
0-CONTROL	100%
5 picograms p	100%
50 pg p	100%
0.5 nano	85%
	(Mean from two expts.)
5 ng	68%
50 ng	63%
500 ng	64%
	(Mean from two expts.)
5 µg	70%

TABLE V

HORMONE LEVELS IN THE RATE CIRCULATION						
ANIMALS TREATED DAYS 3 - 13. ANIMALS SACRIFICED ON DAY 14.						
DOSE	% TUMOUR INHIBITION	FSH (NG/ML)	PROLACTIN (NG/ML)	LH (NG/ML)	TESTO. (NG/ML)	HSPI (NG/ML)
CONT.	0	9.35+/-.92	415+/-194	.68+/-.24	1.4+/-.27	7.48+/-0.5
5NG	32%	4.6+/-.95	273+/-93	.39+/-.03	2.0+/-.67	7.0+/-1.3

TABLE V (continued)

HORMONE LEVELS IN THE RATE CIRCULATION						
ANIMALS TREATED DAYS 3 - 13. ANIMALS SACRIFICED ON DAY 14.						
50 NG HSPI	39%	3.73+/-.36	245+/-70	.30+/-.04	1.1+/-.98	9.39+/-1.0

Claims

1. A composition for use in the medical treatment of benign prostatic hyperplasia, adenocarcinoma of the prostate, breast or gastrointestinal tract, comprising:
in combination an anticancer drug and a peptide selected from human seminal plasma inhibin (Sequence ID No.1) or a decapeptide (Sequence ID No.2), being Tyr-Thr-Cys-Ser-Val-Ser-Glu-Trp-Gly-Ile-OH, wherein the cysteine residue is protected by an acetamidomethyl group.

2. A composition for use in the treatment of diseases characterised by elevated levels of FSH, comprising:
in combination an anticancer drug and a peptide selected from human seminal plasma inhibin (Sequence ID No.1) and a decapeptide (Sequence ID No.2).

3. A composition according to claim 1 or 2 wherein said anticancer drug is selected from mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin and daunomycin.

4. A composition according to claims 1, 2 or 3 comprising a pharmaceutically acceptable delivery means including time release encapsulation means.

Patentansprüche

1. Zusammensetzung zur Verwendung bei der medizinischen Behandlung einer gutartigen prostatischen Hyperplasie, eines Adenokarzinoms der Prostata, der Brust oder des gastrointestinalen Trakts, umfassend:
in Kombination ein Krebsmittel und ein Peptid, ausgewählt aus einem menschlichen Samenplasmahemmstoff (fortlaufende ID-Nr. 1) bzw. einem Dekapeptid (fortlaufende ID-Nr. 2), wobei es sich um Tyr-Thr-Cys-Ser-Val-Ser-Glu-Trp-Gly-Ile-OH handelt, wobei der Zysteinrest durch eine Acetamidmethylgruppe geschützt ist.

2. Zusammensetzung zur Verwendung bei der Behandlung von Krankheiten, gekennzeichnet durch erhöhte FSH-Werte, umfassend:
in Kombination ein Krebsmittel und ein Peptid, ausgewählt aus einem menschlichen Samenplasmahemmstoff (fortlaufende ID-Nr. 1) und einem Dekapeptid (fortlaufende ID-Nr. 2).

3. Zusammensetzung nach Anspruch 1 oder 2, wobei das Krebsmittel aus Mitomycin, Idarubicin, Cisplatin, 5-Fluor-Uracil, Methotrexat, Adriamycin und Daunomycin ausgewählt ist.

4. Zusammensetzung nach Anspruch 1, 2 oder 3, umfassend eine pharmazeutisch zulässige Liefereinrichtung mit einer Zeitfreisetz-Verkapselungseinrichtung.

Revendications

1. Composition pour l'utilisation dans le traitement médical de l'hyperplasie prostatique bénigne, de l'adénocarcinome de la prostate, du sein ou du tractus gastro-intestinal, comprenant:
en combinaison un médicament anticancéreux et un peptide sélectionné dans le poupe constitué par l'inhibine du plasma séminal humain (Séquence ID N°1) ou un décapeptide (Séquence ID N°2), dont la séquence est Tyr - Thr - Cys - Ser - Val - Ser - Glu - Trp - Gly - Ile - OH, où le résidu cystéine est protégé par un groupe acétoamidométhyle.

2. Composition pour l'utilisation dans le traitement de maladies caractérisées par des niveaux élevés de FSH, comprenant:
en combinaison un médicament anticancéreux et un peptide sélectionné dans le groupe constitué par l'inhibine du plasma séminal humain (Séquence ID N°1) et un décapeptide (Séquence ID N°2).

3. Composition selon la revendication 1 ou 2, dans laquelle ledit médicament anticancéreux est sélectionné dans le groupe constitué par la mitomycine, l'idarubicine, la cisplatine, le 5-fluoro-uracile, le méthotrexate, l'adriamycine et la daunomycine.

4. Composition selon les revendications 1, 2 ou 3 comprenant un moyen de délivrance acceptable du point de vue pharmaceutique incluant un moyen d'encapsulation à libération retardée.

NH2-Ser Cys Tyr Phe Ile Pro Asn Glu Gly Val
1 5 10
Pro Gly Asp Ser Thr Arg Lys Cys Met Asp
15 20
Leu Lys Gly Asn Lys His Pro Ile Asn Ser
25 30
Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys
35 40
Thr Cys Tyr Glu Thr Glu Ile Ser Cys Cys
45 50
Thr Leu Val Ser Thr Pro Val Gly Tyr Asp
55 60
Lys Asp Asn Cys Gln Arg Ile Phe Lys Lys
65 70
Glu Asp Cys Lys Tyr Ile Val Val Glu Lys
75 80
Lys Asp Pro Lys Lys Thr Cys Ser Val Ser
85 90
Glu Trp Ile Ile-COOH
94

FIGURE 1

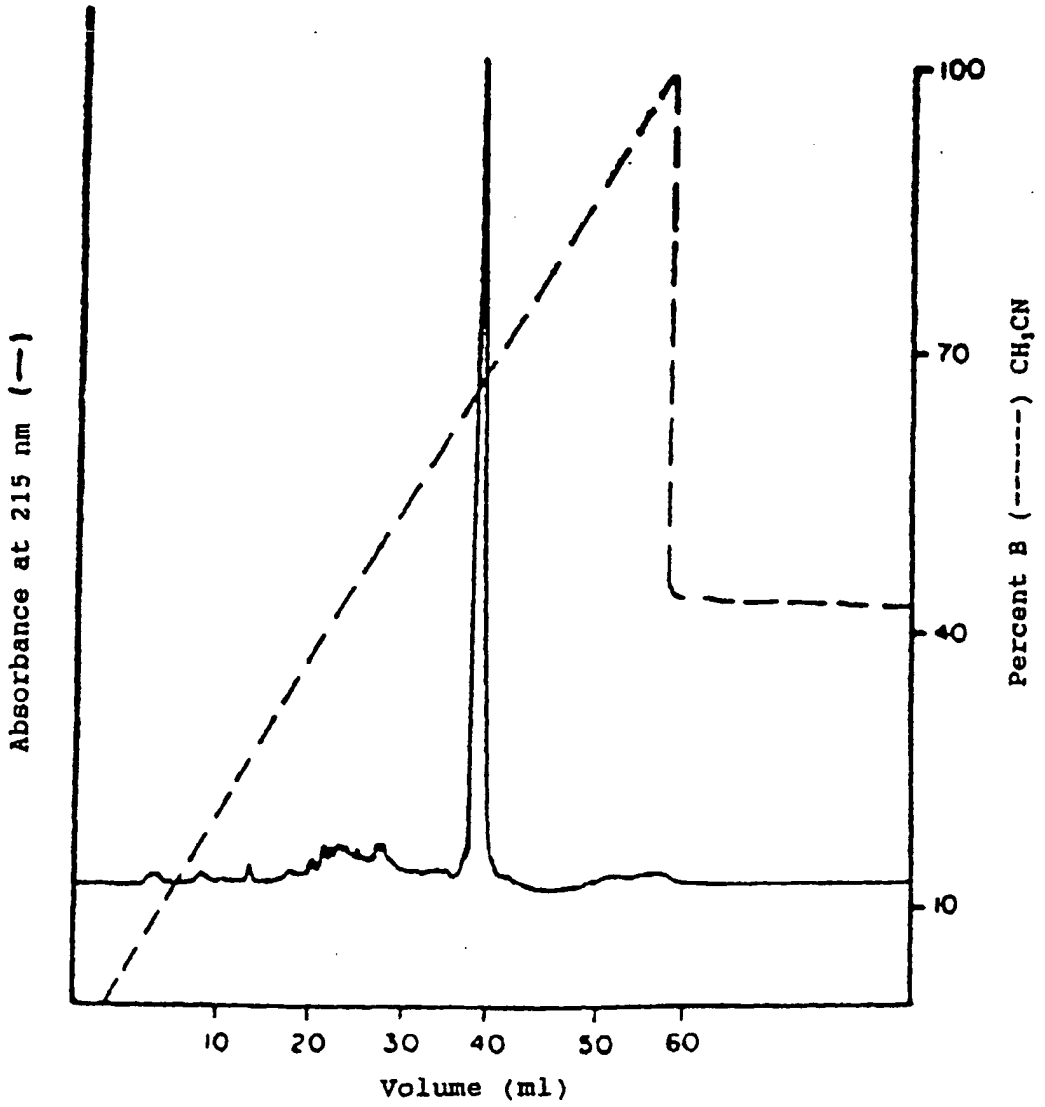
Absorbance at 215 nm (—)
Percent B (------) CH3CN
100
70
40
10
10
20
30
40
50
60
Volume (ml)

FIGURE 2

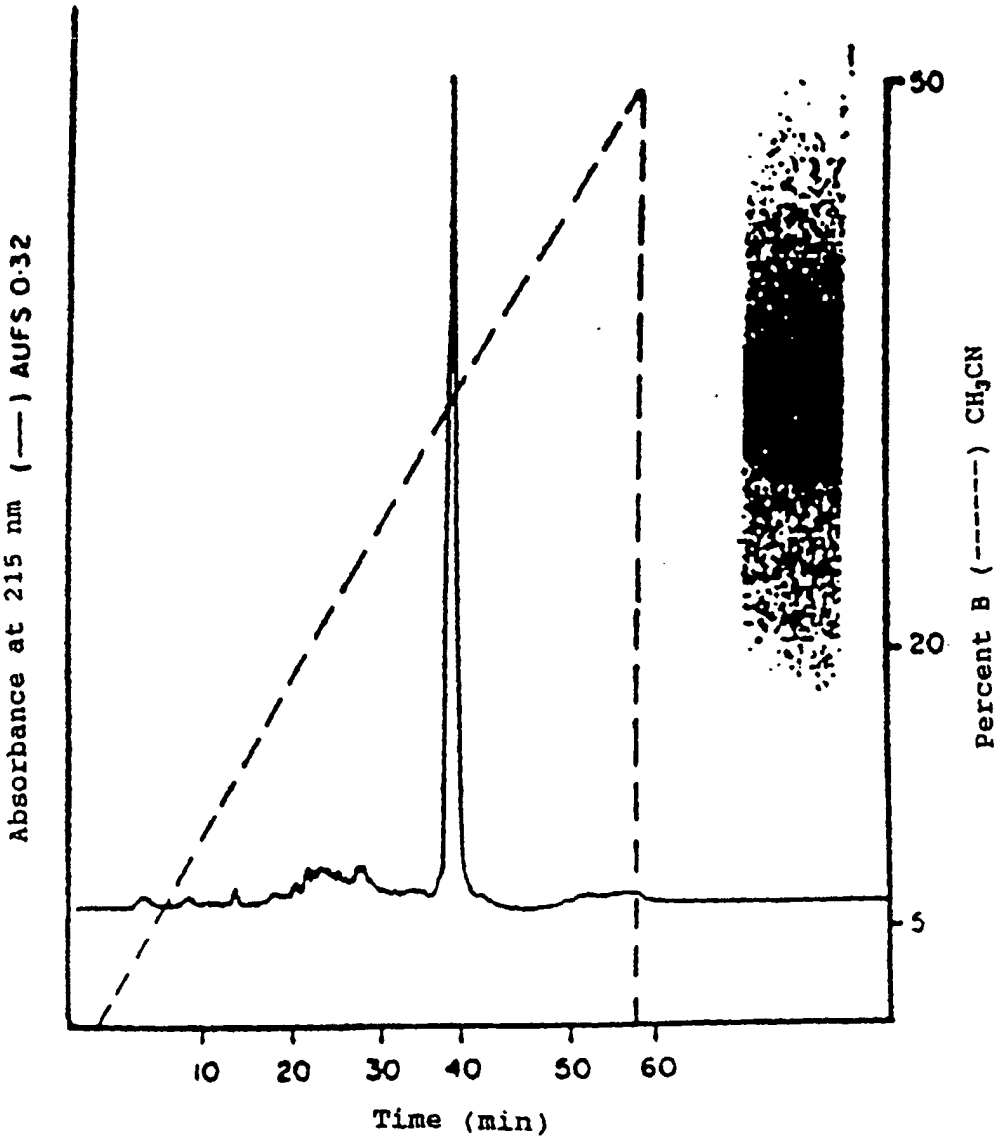
Absorbance at 215 nm (——) AUFS 0·32
Percent B (- - - - - -) CH₃CN
50
20
5
10
20
30
40
50
60
Time (min)

FIGURE 3

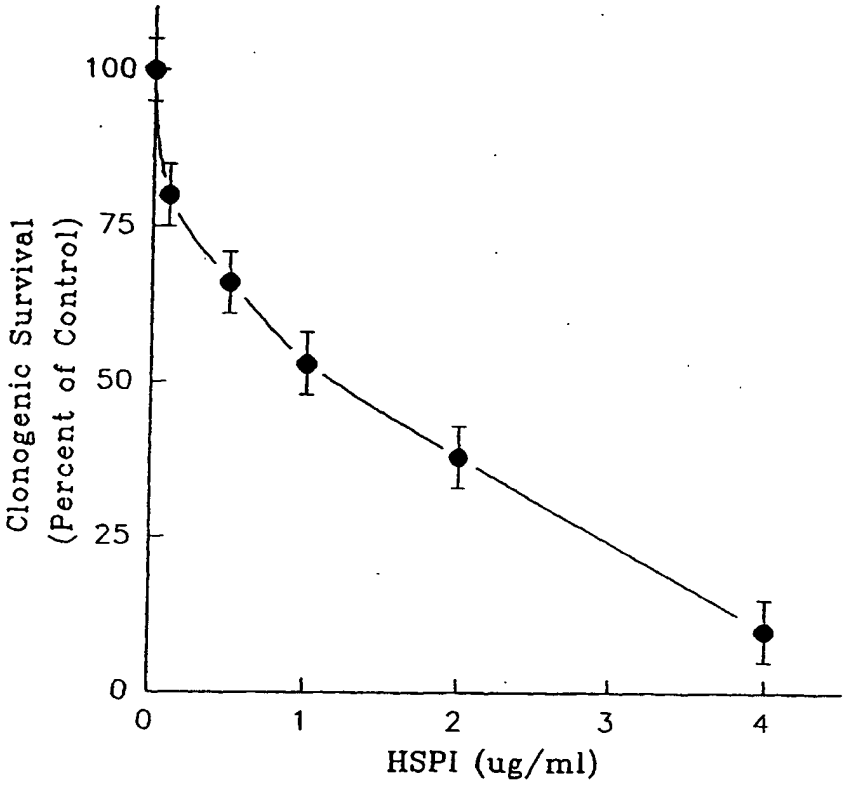
100
75
50
25
0
Clonogenic Survival
(Percent of Control)
0
1
2
3
4
HSPI (ug/ml)

FIGURE 4

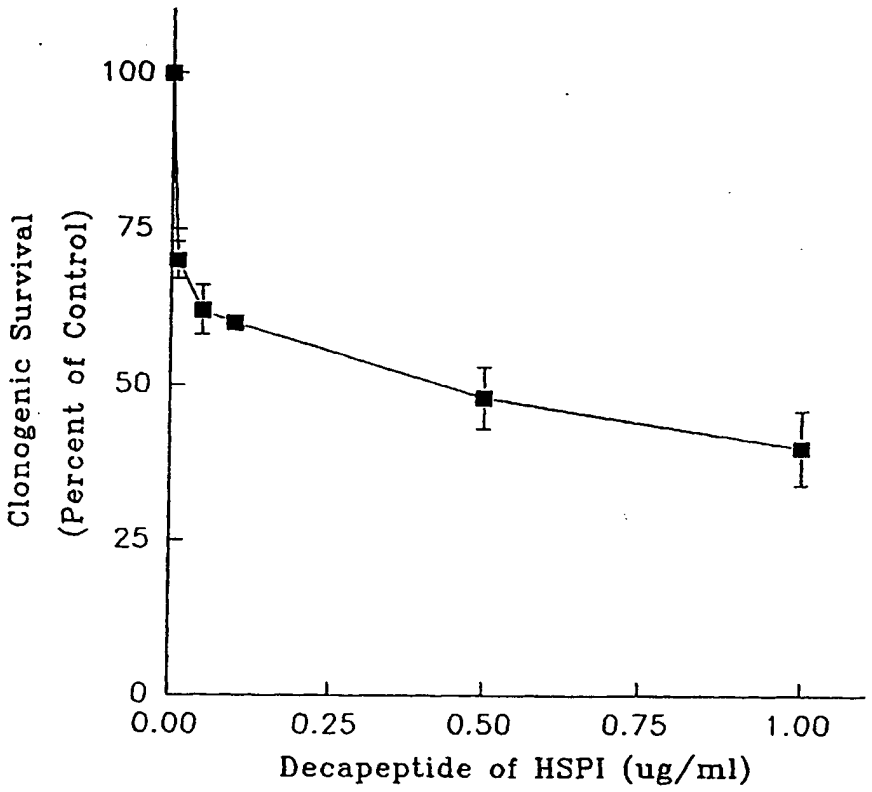
Clonogenic Survival
(Percent of Control)
100
75
50
25
0
0.00
0.25
0.50
0.75
1.00
Decapeptide of HSPI (ug/ml)

FIGURE 5

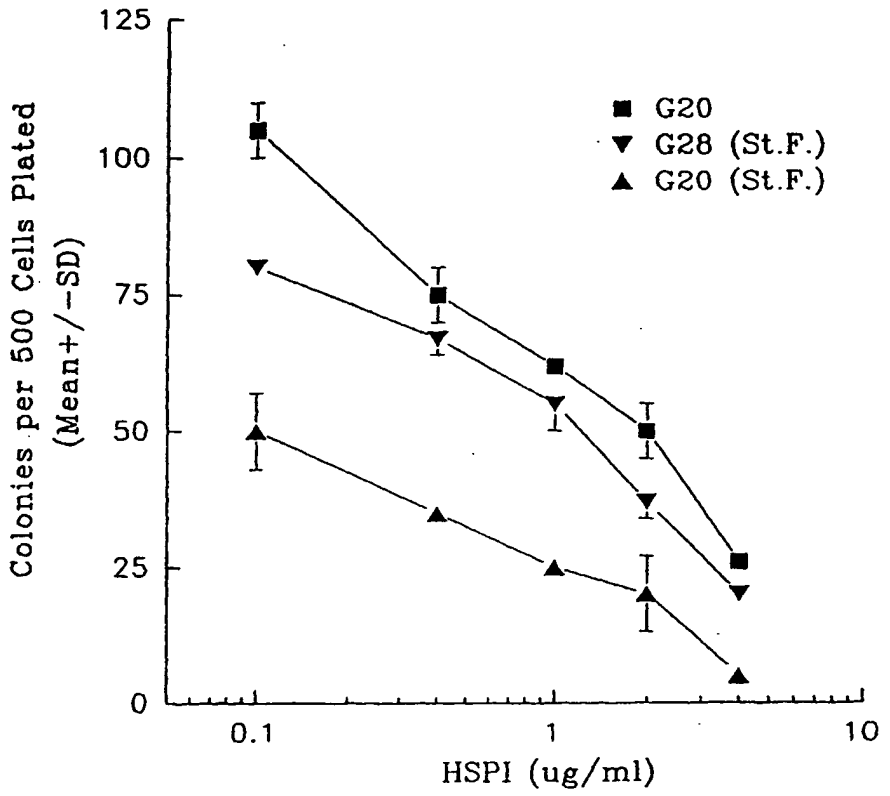
125
100
75
50
25
0
Colonies per 500 Cells Plated
(Mean+/-SD)
G20
G28 (St.F.)
G20 (St.F.)
0.1
1
10
HSPI (ug/ml)

FIGURE 6

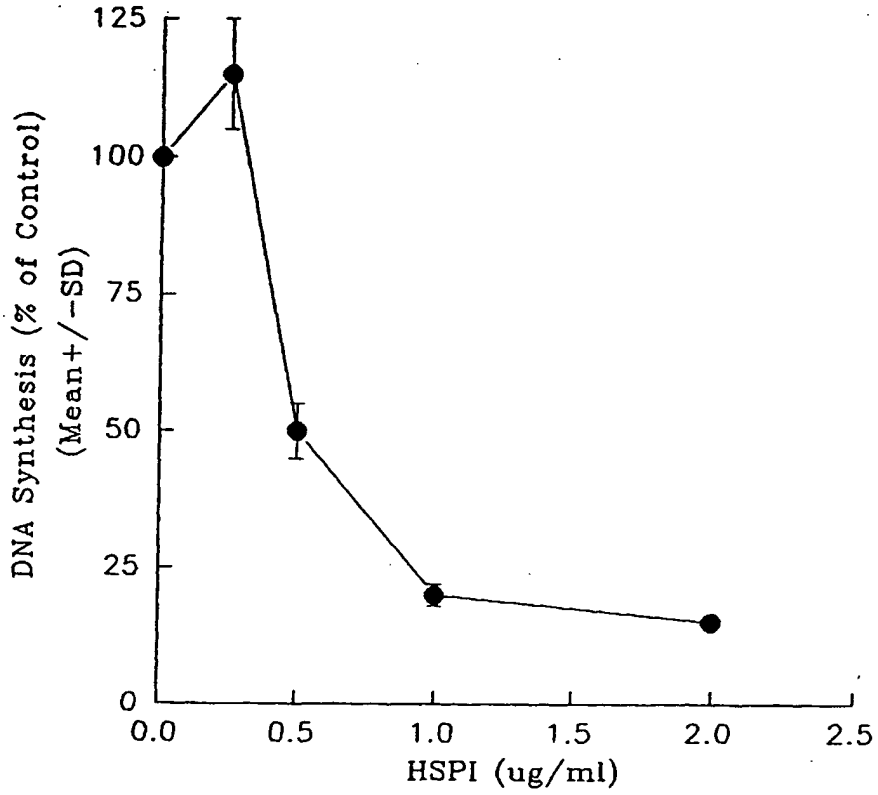
125
100
75
50
25
0
DNA Synthesis (% of Control)
(Mean+/-SD)
0.0
0.5
1.0
1.5
2.0
2.5
HSPI (ug/ml)

FIGURE 7

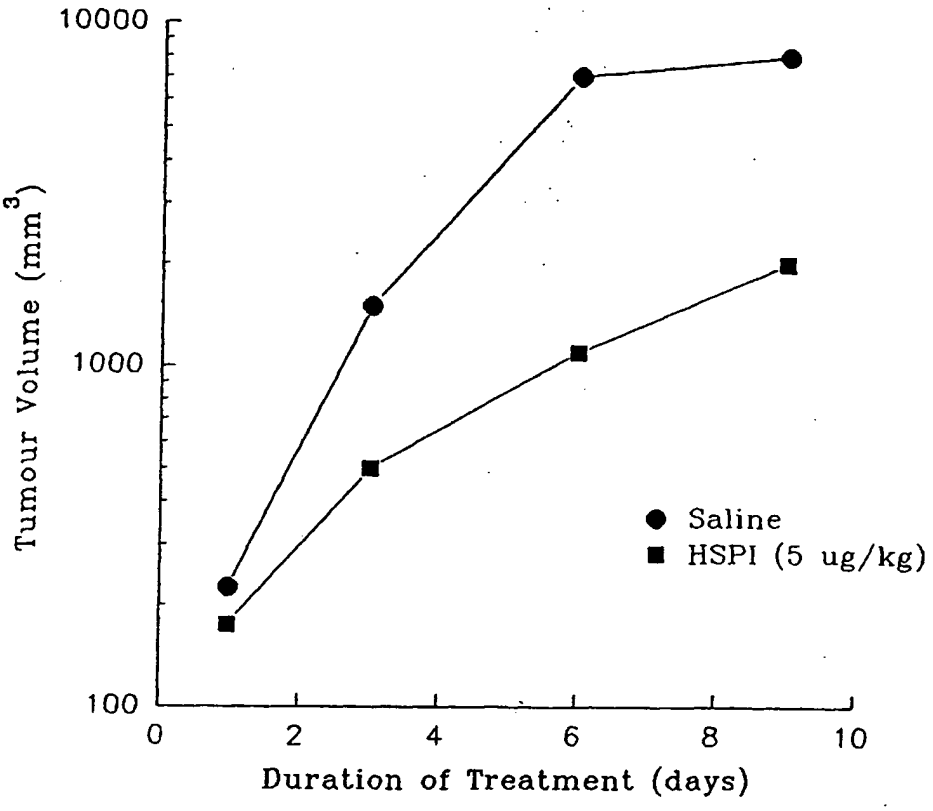
10000
1000
100
Tumour Volume (mm^3)
0
2
4
6
8
10
Duration of Treatment (days)
Saline
HSPI (5 ug/kg)

FIGURE 8

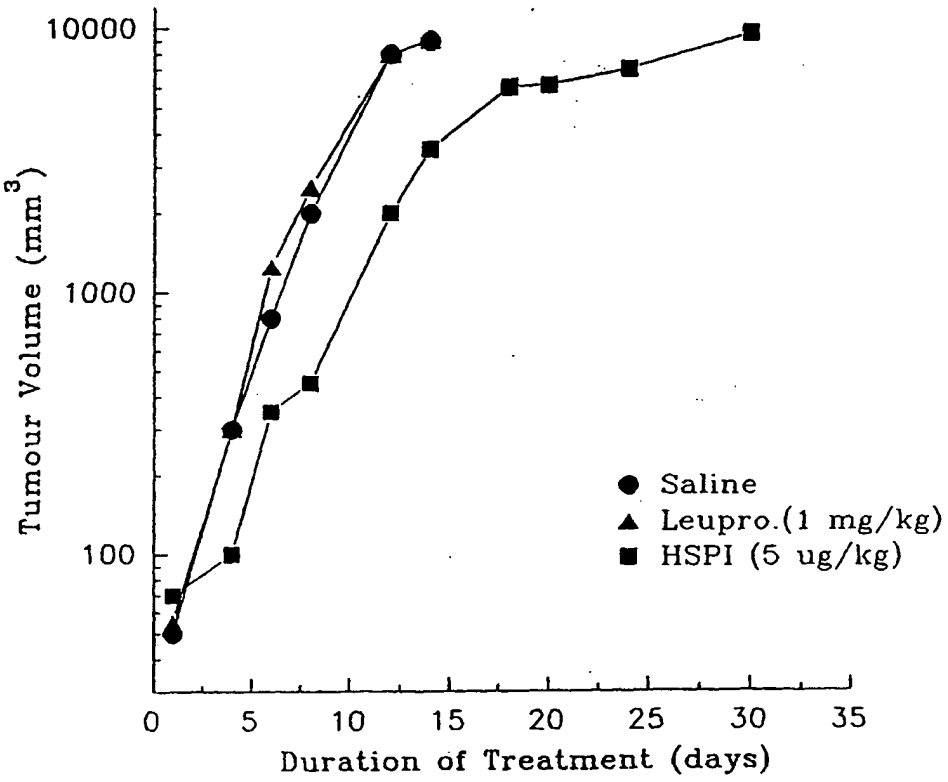
10000
1000
100
Tumour Volume (mm^3)
0
5
10
15
20
25
30
35
Duration of Treatment (days)
Saline
Leupro.(1 mg/kg)
HSPI (5 ug/kg)

FIGURE 9

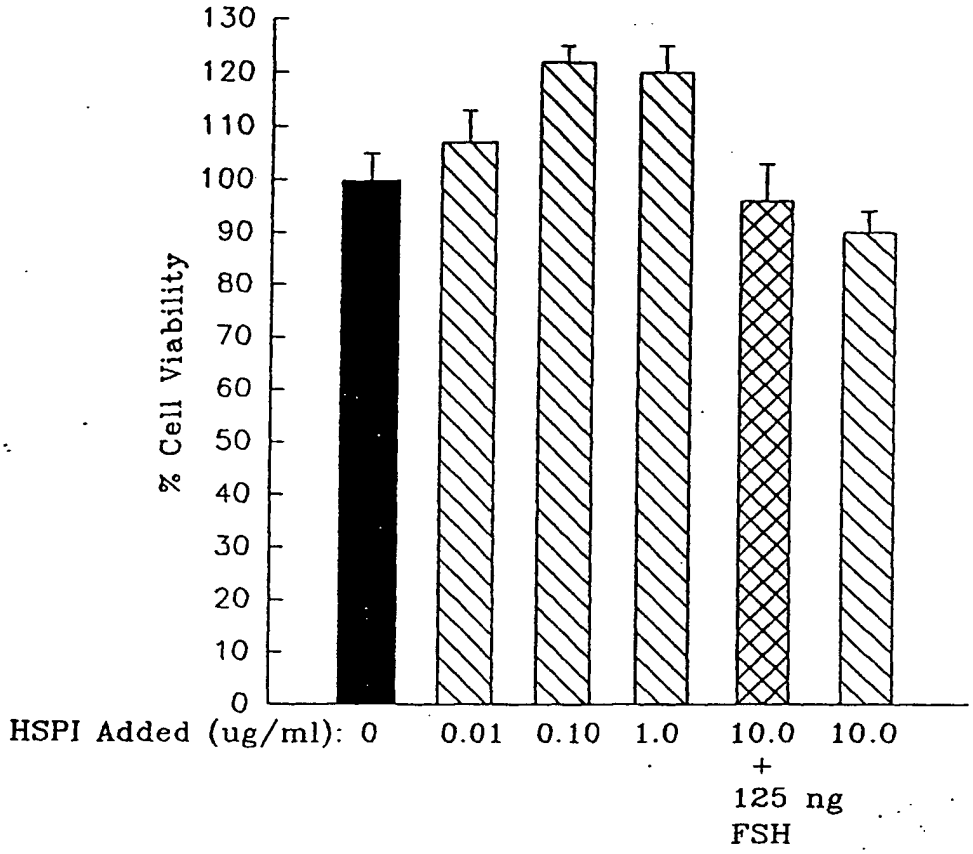
130
120
110
100
90
80
70
60
50
40
30
20
10
0
% Cell Viability
HSPI Added (ug/ml): 0
0.01
0.10
1.0
10.0
10.0
+
125 ng
FSH

FIGURE 10

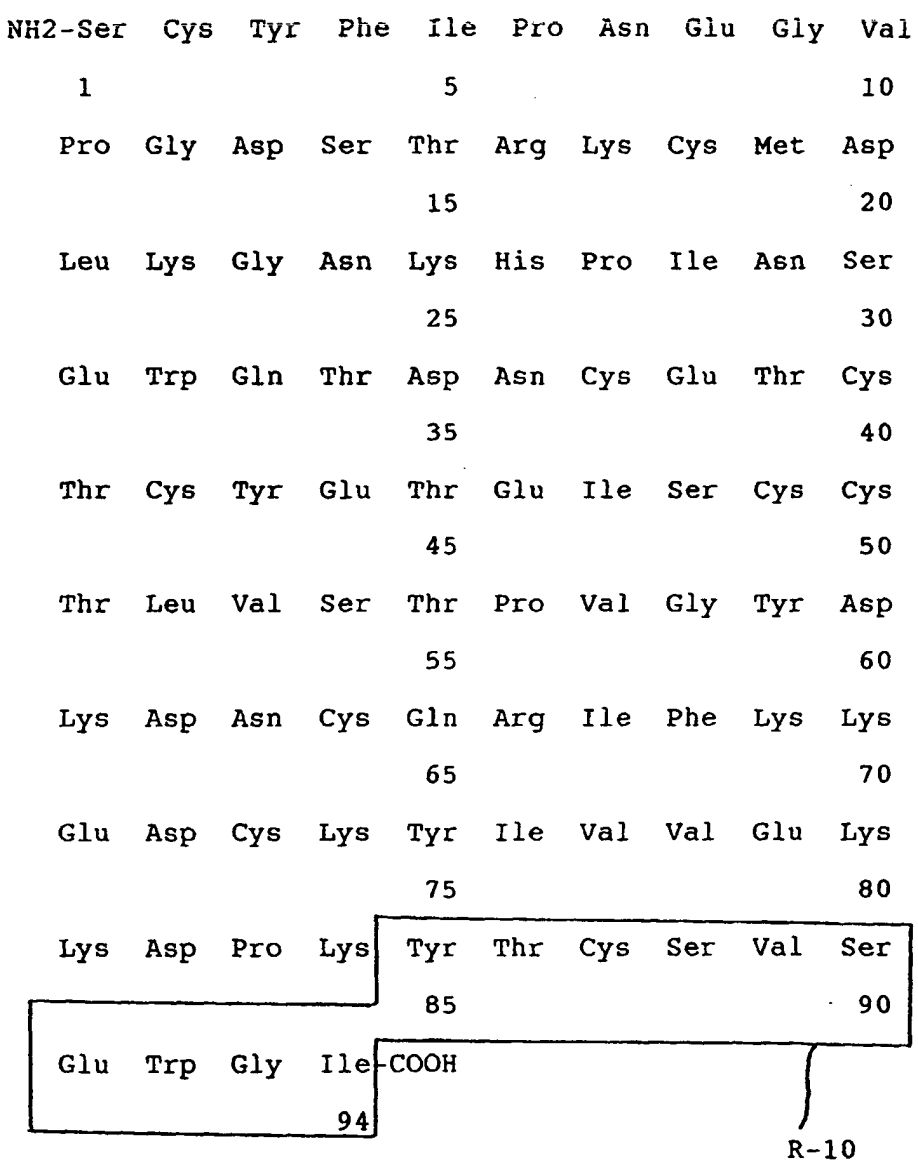
NH2-Ser Cys Tyr Phe Ile Pro Asn Glu Gly Val
1 5 10
Pro Gly Asp Ser Thr Arg Lys Cys Met Asp
15 20
Leu Lys Gly Asn Lys His Pro Ile Asn Ser
25 30
Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys
35 40
Thr Cys Tyr Glu Thr Glu Ile Ser Cys Cys
45 50
Thr Leu Val Ser Thr Pro Val Gly Tyr Asp
55 60
Lys Asp Asn Cys Gln Arg Ile Phe Lys Lys
65 70
Glu Asp Cys Lys Tyr Ile Val Val Glu Lys
75 80
Lys Asp Pro Lys Tyr Thr Cys Ser Val Ser
85 90
Glu Trp Gly Ile-COOH
94
R-10

FIGURE 11

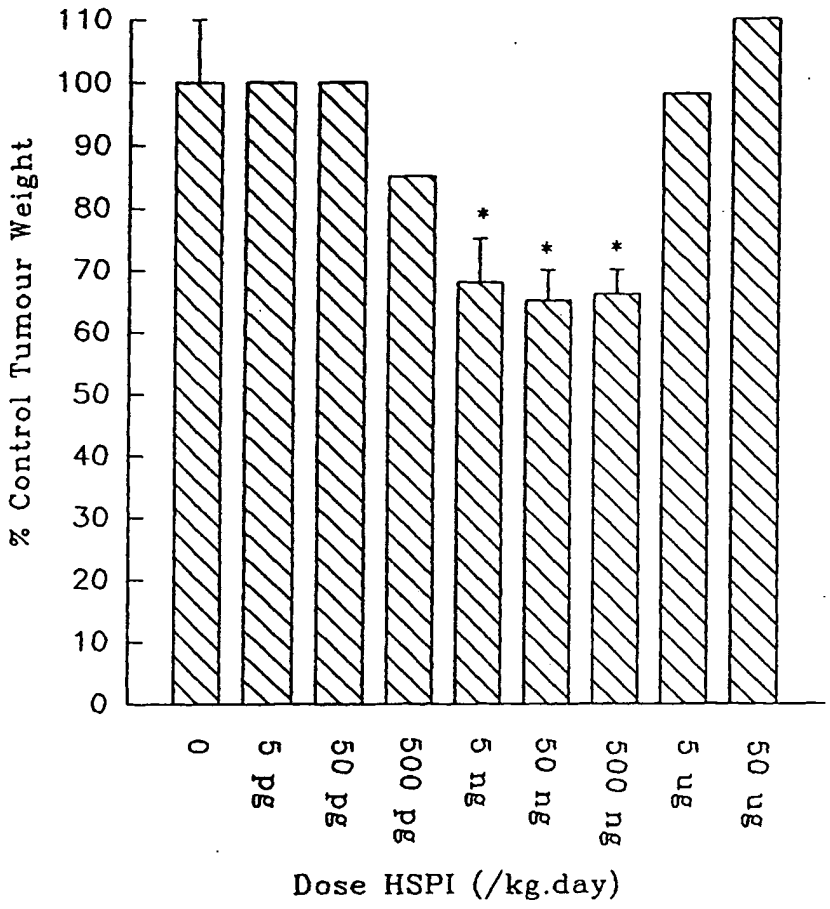
% Control Tumour Weight
110
100
90
80
70
60
50
40
30
20
10
0
*
*
*
0
5 pg
50 pg
500 pg
5 ng
50 ng
500 ng
5 ug
50 ug
Dose HSPI (/kg.day)

FIGURE 13

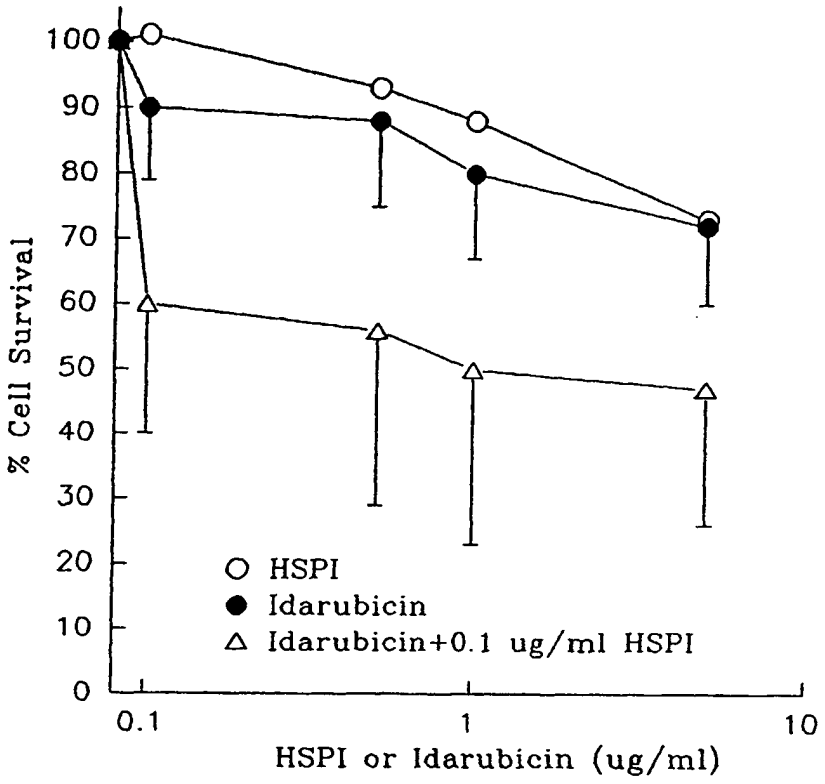
100
90
80
70
60
50
40
30
20
10
0
% Cell Survival
HSPI
Idarubicin
Idarubicin+0.1 ug/ml HSPI
0.1
1
10
HSPI or Idarubicin (ug/ml)

FIGURE 14

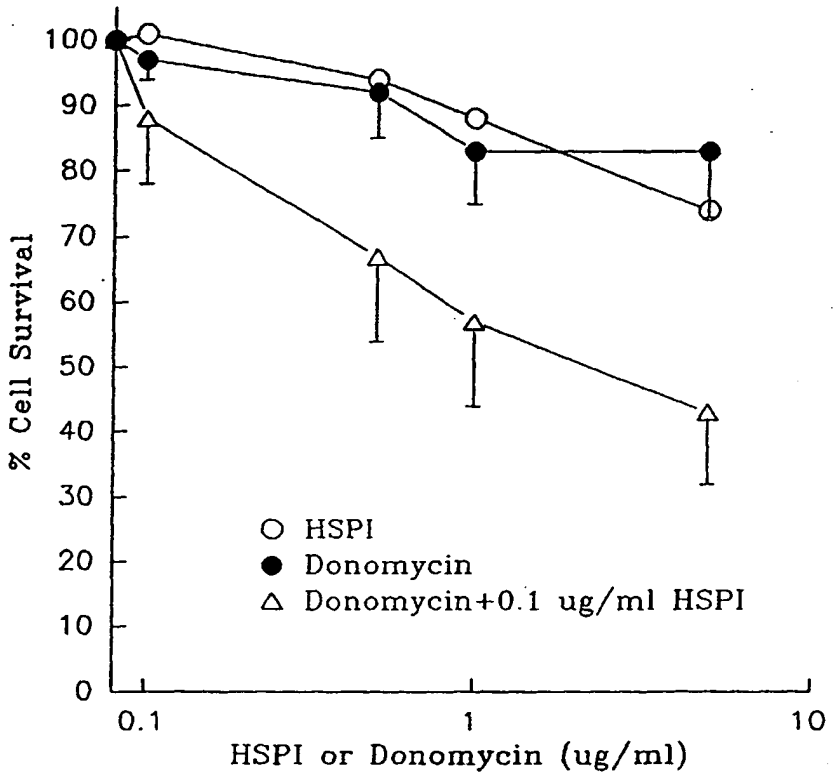
100
90
80
70
60
50
40
30
20
10
0
% Cell Survival
0.1
1
10
HSPI or Donomycin (ug/ml)
HSPI
Donomycin
Donomycin+0.1 ug/ml HSPI

FIGURE 15

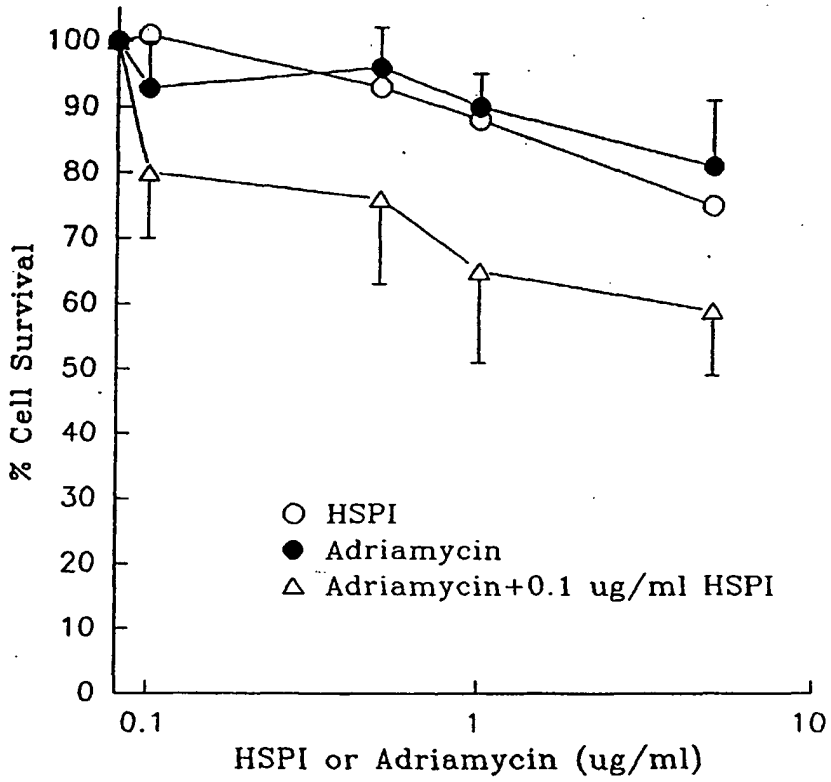
100
90
80
70
60
50
40
30
20
10
0
% Cell Survival
0.1
1
10
HSPI or Adriamycin (ug/ml)
HSPI
Adriamycin
Adriamycin+0.1 ug/ml HSPI

FIGURE 16

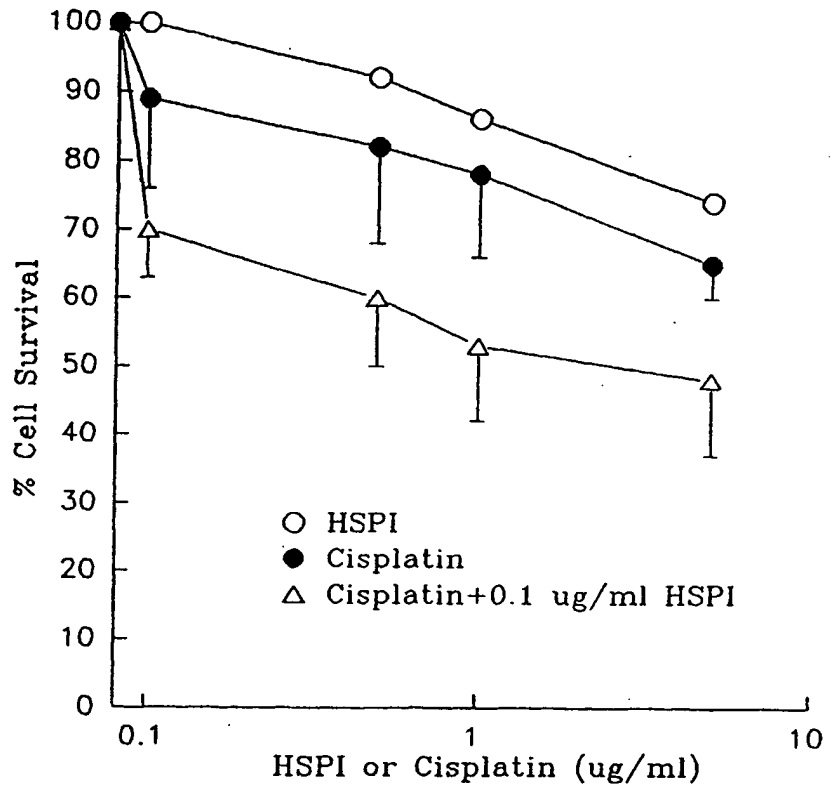
100
90
80
70
60
50
40
30
20
10
0
% Cell Survival
HSPI
Cisplatin
Cisplatin+0.1 ug/ml HSPI
0.1
1
10
HSPI or Cisplatin (ug/ml)

FIGURE 17

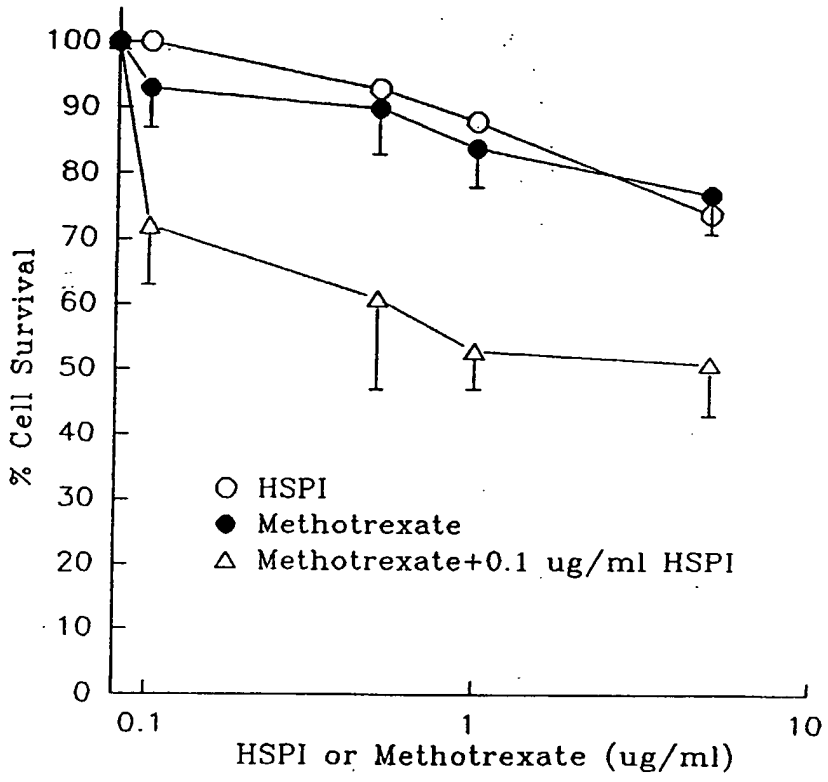
100
90
80
70
60
50
40
30
20
10
0
% Cell Survival
0.1
1
10
HSPI or Methotrexate (ug/ml)
HSPI
Methotrexate
Methotrexate+0.1 ug/ml HSPI

FIGURE 18

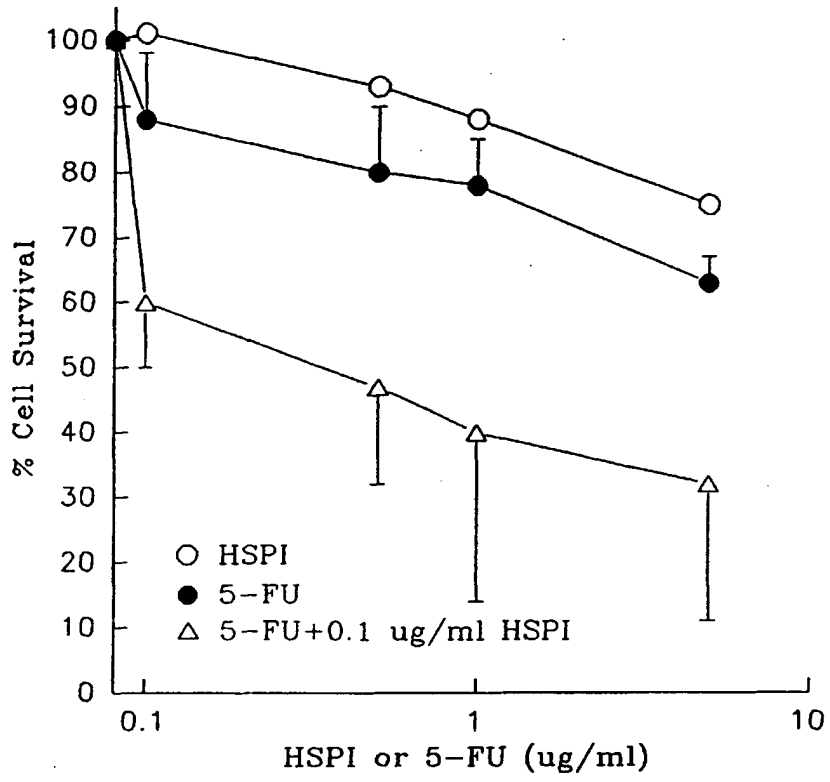
100
90
80
70
60
50
40
30
20
10
0
% Cell Survival
HSPI
5-FU
5-FU+0.1 ug/ml HSPI
0.1
1
10
HSPI or 5-FU (ug/ml)

FIGURE 19

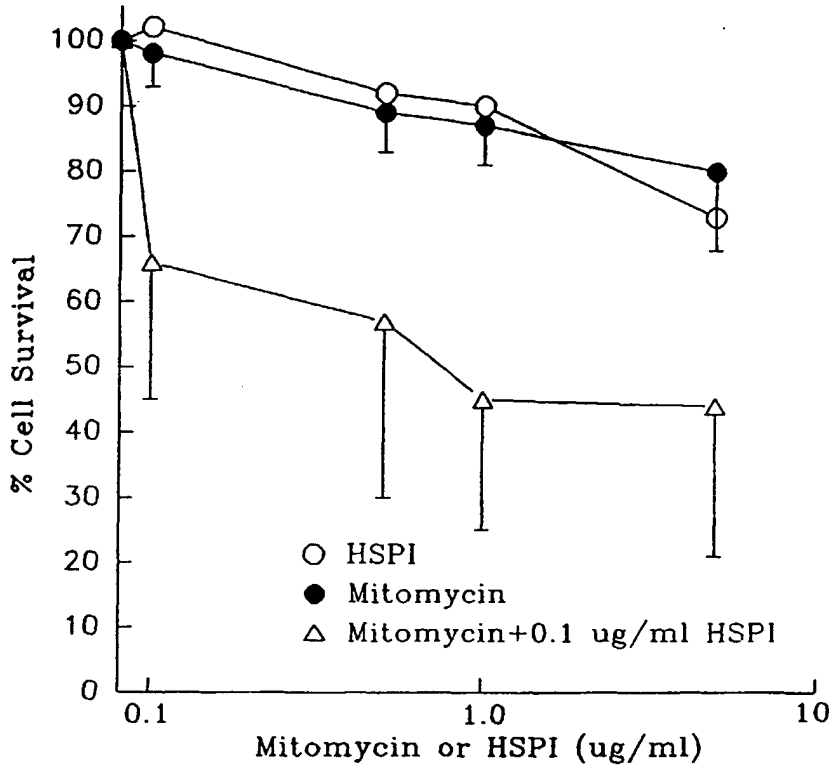
100
90
80
70
60
50
40
30
20
10
0
% Cell Survival
HSPI
Mitomycin
Mitomycin+0.1 ug/ml HSPI
0.1
1.0
10
Mitomycin or HSPI (ug/ml)

FIGURE 20